Strain Dependence of the Elastic Properties of Force-Producing Cross-Bridges in Rigor Skeletal Muscle

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ABSTRACT Stretch and release experiments carried out on skinned single fibers of frog skeletal muscle under rigor conditions indicate that the elastic properties of the fiber depend on strain. For modulation frequencies below 1000 Hz, the results show an increase in Young's modulus of 20% upon a stretch of 1 nm/half-sarcomere. Remarkably, the strain dependence of Young's modulus decreases at higher frequencies to about 10% upon a 1-nm/half-sarcomere stretch at a modulation frequency of 10 kHz. This suggests that the cause of the effect is less straightforward than originally believed: a simple slackening of the filaments would result in an equally large strain dependence at all frequencies, whereas strain-dependent properties of the actin filaments should show up most clearly at higher frequencies. We believe that the reduction of the strain dependence points to transitions of the cross-bridges between distinct force-producing states. This is consistent with the earlier observation that Young's modulus in rigor increases toward higher frequencies

INTRODUCTION

Tension in muscle is generated by cross-bridges protruding from the myosin filament and binding to the actin filament (Huxley, 1957; Huxley and Simmons, 1971). The cross-bridges cycle through force-producing, strongly bound and non-force-producing, weakly bound states. The transitions between these states are correlated to the hydrolysis of ATP, which provides the free energy for the mechanical power stroke (Kushmerick and Davies, 1969; Lymn and Taylor, 1971).

The actomyosin complex is in a force-producing state in the absence of nucleotide (AM) or when ADP is bound to the myosin (AM-ADP). Whereas the AM-ADP state is considered the principal force-producing state, model studies suggest that the free energies of the AM and AM-ADP states are quite similar (Pate and Cooke, 1989; Smith and Geeves, 1995). Therefore, we believe that the rigor state is particularly convenient for studying the mechanical properties of the force-producing cross-bridges. In the absence of ATP, Mg^{2+} and Ca^{2+}, the cross-bridge cycling is inhibited and virtually all of the cross-bridges are strongly bound to the actin filament.

In earlier studies quick stretch-and-release experiments were carried out to characterize the elastic properties of rigor skeletal muscle of the frog in the frequency range from 250 Hz to 50 kHz (Jung et al., 1988; De Winkel et al., 1993). The results show a strong frequency dependence of the complex Young's modulus Z, which is related to the viscoelastic properties of the half-sarcomere as $Z \times l/A$, where l is the length of the half-sarcomere, and A is the cross-sectional area of the preparation. An additional result from the stretch-and-release experiments was that the Young's modulus obtained from a stretch was consistently larger than that obtained from a release (Jung et al., 1988) (Fig. 1). This points to a strain dependence of the elastic properties, but it is not possible to extract detailed information on the size and frequency characteristic of the strain dependence from these data.

Here we consider the strain dependence of Young's modulus in greater detail. A skinned fiber of the M. iliofibularis of the frog was mounted between a loudspeaker motor and a force transducer. The tension response to a stepwise length change was subtracted from the tension response to the superposition of an identical step and a sinusoidal modulation in the frequency range from 250 Hz to 10 kHz. The amplitude of the resulting sinusoidal transient was found to change rapidly after the length step. For eleminating experimental factors such as the length, radius, and mass density of the preparation and the frequency characteristics of the experimental set-up, we developed a method of extracting Young's modulus at the modulation frequency as a function of time.

The time-resolved Young's modulus thus obtained is found to increase within 0.5 ms after a stretch and remains constant thereafter. A rapid decrease is observed after a release. At frequencies below 1 kHz the relative change in the Young's modulus is about 20% per nm/half-sarcomere stretch or release. Indeed, evidence for such a strain dependence of the rigor elasticity was found in other studies and is considered a characteristic property of the rigor muscle (Kawai and Brandt, 1976; Podolsky et al., 1982; Higuchi et al., 1995). Remarkably, our results show that the size of the effect decreases toward higher frequencies: at 10 kHz Young's modulus only changes 10% per nm/half-sarcomere stretch or release.

Originally, several explanations were given for the strain dependence of the rigor stiffness. Assuming that the number

Received for publication 16 April 1996 and in final form 18 October 1996.
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0006-3495/97/02/814/08 $2.00

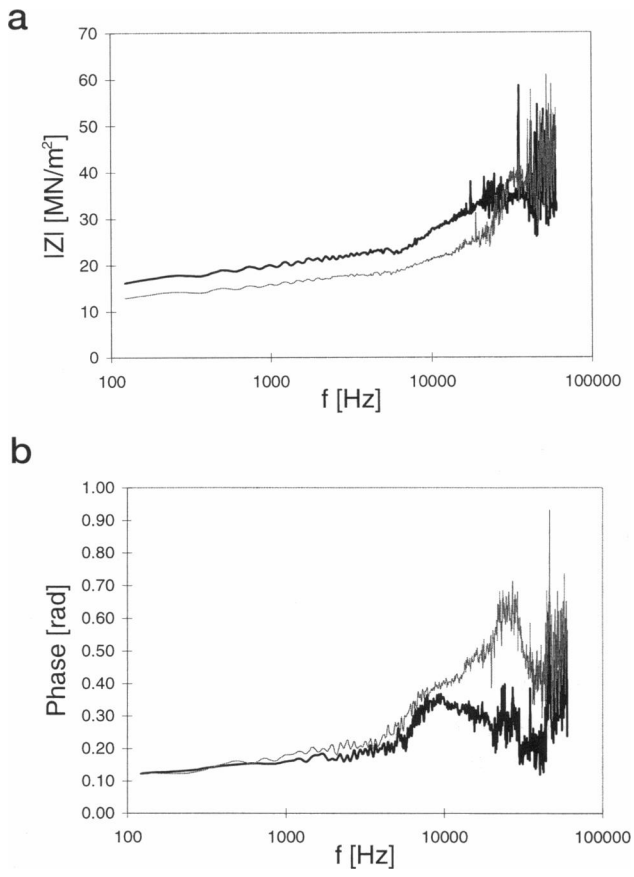

FIGURE 1 The Young's modulus of the muscle fiber under rigor conditions as obtained from a stretch of 1 nm/half-sarcomere (thick) and a release of 1 nm/half-sarcomere (thin). (*a*) The norm $|Z|$. (*b*) The phase Φ_Z.

of cross-bridges does not change in rigor, the change in elasticity could result from slackening of the filaments in the sarcomere (Kawai and Brandt, 1976). In their recent study Higuchi et al. (1995) suggest that the actin filament exhibits a strain-dependent compliance. However, our observation that the strain dependence becomes smaller toward higher frequencies suggests that the cause of the effect is less straightforward. A simple slackening of the filaments in the sarcomere as the result of a release would lead to a loss of stiffness at all frequencies. Strain dependence of the actin filament compliance also fails to explain the reduction of the effect toward higher frequencies. Precisely at high frequencies the contribution of the filament compliance to the total fiber elasticity is large (Blangé et al., 1996). Thus any strain dependence of the filament compliance would show up most clearly at higher frequencies and thus result in an increase of the effect rather than a decrease.

In combination with the earlier finding that Young's modulus increases toward higher frequencies, the reduction of strain dependence points to a complex behavior of the strongly bound cross-bridges in the muscle fiber. In the final section of this paper we discuss various possibilities that may bring about the observed frequency and strain dependence of the elasticity.

MATERIALS AND METHODS

Preparations and solutions

Single muscle fibers of the frog (*Rana esculenta*) were skinned by freeze drying as decribed previously (Stienen et al., 1983; Stienen and Blangé, 1985). A comparison between freeze dried and chemically skinned (Triton X-100) fibers showed that in the relaxed state the chemically skinned fibers were somewhat less stiff. In contrast, in the activated and rigor states no difference was found between the preparations (Jung et al., 1988). This indicates that freeze-dried fibers are suitable for accurately measuring the mechanical stiffness at a microscecond time resolution (De Winkel et al., 1994).

In a skinned muscle fiber rigor can be induced by removal of ATP from the intracellular medium. The addition of ATP will cause the fiber to relax. The lengths of the single muscle fibers ranged from 3 to 7 mm, and the diameters varied between 70 and 200 μm. The composition of the solutions was calculated using a computer program based on that of Fabiato and Fabiato (1979). The composition of the relaxation solution was as follows: imidazol 60 mM; EGTA 20 mM; creatine phosphate 10 mM; MgATP 5 mM; Mg^{2+} 1 mM; Na^+ 31 mM; K^+ 45 mM; Cl^- 51 mM and 50 units/ml creatine kinase. The activation solution was identical to the relaxation solution, except for: Ca^{2+} 0.1 mM; K^+ 46 mM, and Cl^- 53 mM. The composition of the rigor solution was as follows: imidazol 60 mM; EGTA 20 mM; EDTA 5 mM; Na^+ 30 mM; K^+ 52 mM; Cl^- 72 mM. For all solutions the pH was set to 7.0 bij addition of KOH and to an ionic strength of 160 mM by addition of KCl. The temperature of the solutions was kept at 4°C (Jung et al., 1988; De Winkel et al., 1994).

Apparatus

The mechanical stiffness was measured using an apparatus based on that described by Van den Hooff et al. (1982) and Stienen and Blangé (1985). The fiber was mounted isometrically between the center rod of a loudspeaker motor and a homemade force transducer. The motor affords a stepwise change of the fiber length of up to 50 μm within 35 μs. Furthermore, a 10-μm sinusoidal modulation can be realized at frequencies of up to 10 kHz. Low-frequency off-axis oscillations were suppressed by three loops of a thin rope wound around the center rod of the loudspeaker motor. The thin ropes were put under a moderate stress in three directions in the plane perpendicular to the fiber axis.

The force transducer used in the experiments exhibited a resonance frequency of 70 kHz in air, a damping constant of 500 μs, and a noise of 2 μV peak to peak. When a muscle fiber is attached to the transducer and immersed in the buffer solution, the resonance frequency decreases by 5 to 10%. The bandwidth of the force transducer amplifier is 150 kHz (De Winkel et al., 1993).

HeNe laser diffraction was used to check if the applied length change was absorbed by the fiber in the rigor state using the method described previously (De Winkel et al., 1994). The change in position of the first-order laser diffraction line after application of a length step was detected by a position-sensitive photodiode (SD-1166-21-11-391; Silicon Detector Corp., Newbury Park, CA). From this signal the change in sarcomere length was calculated and compared to the ratio of the length change of the motor signal to the total fiber length.

The tension responses and length changes were monitored using a 12-bit ADC, at a sample frequency of 500 kHz (NBMIO16L; National Instruments, Austin, TX), and a microcomputer. As the input signals were filtered at a cutoff frequency of 150 kHz, aliasing at this sample rate was avoided. When a lower sample frequency was required, this 500-kHz signal was digitally filtered afterward to prevent aliasing at lower frequencies.

Experimental protocol

The fibers were mounted isometrically in the dry state between the loudspeaker motor and the homemade force transducer with α-cyanoacrylate

glue (Cyanolit 202; 3 M Center, St. Paul, MN). The Young's modulus of the glued fiber in air was estimated by a sinusoidal analysis of a fiber that was incubated in the glue and subsequently dried. At 25 Hz Young's modulus was found to be 95 ± 5 MN/m^2. This brings us to the conclusion that the compliance of the glued ends in our preparation is negligible and that a sharp interface is obtained between the fiber ends and the functional segment. After the fiber was mounted, it was incubated in the relaxation solution for at least 10 min. The sarcomere length was set to 2.15 μm, and the uniformity of the fiber was checked using helium-neon laser diffraction (HeNe laser Uniphase, model 105-2). Fibers exhibiting nonuniformities larger than ±0.10 μm were discarded (less than 20%). The fiber length and diameter were measured by means of a microscope (Zeiss Opmi-1; f 50 mm for measuring the diameter, f 175 mm for the length, oculars 20×). The fiber was discarded when it was twisted or when visible nonuniformities were found. Before the rigor experiments, the fiber was maximally activated and the tension development recorded. After activation the fiber was relaxed for at least 10 min. Then the muscle fiber was brought into the rigor state by incubating it in the rigor solution. Stiffness experiments were carried out only after the development of a steady rigor tension level. The rigor tension was found to be 30 ± 4 kN/m^2, i.e., about 30% of the tension at maximum activation.

In many studies the length of the sarcomeres is controlled using a striation follower, which monitors the striation in a small segment of the fiber and feeds the signal back to the loudspeaker motor. However, this approach cannot be used in the high frequency range (Ford et al., 1977). The distance between the segment and the motor introduces a phase shift of about 7 μs/mm (corresponding to a velocity of sound of 150 m/s in the preparation). As a consequence, a correction of the segment length only reaches the segment after some time. This makes a proper length control of a step realized in 35 μs impossible. Similar reasoning shows that this approach does not eliminate the influence of inhomogeneities in the preparation outside the chosen segment. The distance between the segment and the force transducer results in a phase shift between the segment length change and the tension response. This must be taken into account for a correct determination of the mechanical transfer impedance and subsequently the Young's modulus. Importantly, the phase shift depends on the velocity of sound and thus the Young's modulus in the preparation between the segment and the force transducer. Any inhomogeneities in this section influence the observed phase shift and thus may result in a wrong value for Young's modulus in the kilohertz range.

For these reasons we have chosen to use length-controlled experiments over the full frequency range between 250 Hz and 40 kHz. HeNe laser diffraction was used to check that the applied length change was absorbed by the sarcomeres. As an additional check we measured the tension responses to a rapid stretch and release of the fiber in relaxation and rigor (Jung et al., 1988). In the simulation of the tension response the fiber length was included as an extra variable parameter. In particular, the initial oscillation in the tension response (Fig. 3 *b*) is quite sensitive to the fiber length. Any discrepancies between the fiber lengths obtained in this way would indicate that the model simulation was inadequate because of inhomogeneity of the fiber or compliances of the fiber ends. The fibers exhibited a discrepancy of less than 10%.

The time-resolved Young's modulus

For characterizing the strain dependence of the elastic properties of the muscle fiber, three length modulations were applied and the resulting tensions recorded: 1) the tension response to a length step of up to 4 nm/half-sarcomere (T_{step}); 2) the tension response to an identical step modulated with a sine of 0.25 nm/half-sarcomere top-top, at frequencies ω_{mod} between 250 and 10,000 Hz ($T_{step+sin}$); 3) the tension response to the same signal as 2), but with a 90° phase shift of the sine relative to the step ($T_{step+cos}$). From T_{step} the frequency dependence of the complex Young's modulus can be derived (Blangé and Stienen, 1985; De Winkel et al., 1993). The combination of the three responses affords the extraction of Young's modulus at the modulation frequency as a function of time, during the length step. To this end, the net tension responses to the oscillations are obtained as $T_s = T_{step+sin} - T_{step}$ and $T_c = T_{step+cos} - T_{step}$. The linearity of the apparatus was checked using a silk fiber at a resting tension similar to that of a rigor muscle fiber. The net displacement oscillation (frequency 10 kHz) showed no change in amplitude during a stretch or release.

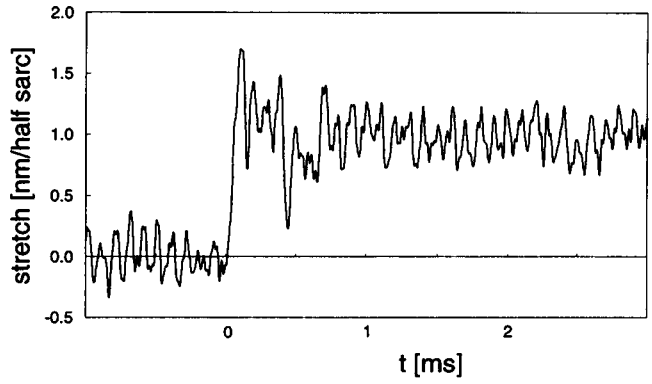

FIGURE 2 The length change of the sarcomeres as observed by HeNe diffraction upon application of a 1 nm/half-sarcomere stretch plus a 10-kHz oscillation of about 0.4 nm/half-sarcomere peak to peak.

DATA ANALYSIS

Extraction of Young's modulus from a stepwise stretch

The complex Young's modulus $Z(s)$ of an infinitesimally small segment of the muscle fiber is calculated from the transfer impedance $H(s)$, which is defined as the ratio of the tension response and the relative length change (De Winkel et al., 1993):

$$H(s) = \frac{T(s)}{\epsilon(s)} = (-2kl)\frac{f(s) \cdot Z(s)}{\exp(kl) - \exp(-kl)}, \qquad (1)$$

with $s = i\omega$ the angular frequency, $i = \sqrt{(-1)}$, $T(s)$ is the Laplace transform of the tension (i.e., the force divided by the cross-sectional area of the fiber), and $\epsilon(s)$ is the Laplace transform of the applied relative length change. l is the fiber length, and $f(s)$ represents the frequency characteristics of the force transducer, the power amplifier (cutoff frequency 150 kHz), and the displacement detector. Although it is a complex quantity, k is denoted as a wave number and is defined as

$$k^2 = \frac{\rho_f \cdot s^2 + (4\rho_m\eta R^{-2})^{0.5}s^{1.5} + \eta R^{-2}s}{Z(s)}, \qquad (2)$$

where ρ_f is the fiber density, ρ_m and η are the density and viscosity of the surrounding fluid, and R is the radius of the fiber. This equation incorporates a correction for the viscous drag (Blangé and Stienen, 1985).

Young's modulus Z cannot be solved simply from these two equations. However, when a good approximation of the complex wave number k is known, Z can be recovered from the data as follows. The derivative of H with respect to Z is

$$\frac{dH(s)}{dZ(s)} = \frac{H(s)}{2Z(s)}\left(1 + kl\frac{\exp(kl) + \exp(-kl)}{\exp(kl) - \exp(-kl)}\right). \qquad (3)$$

For small changes we find

$$\frac{\Delta Z}{Z} = \frac{2}{\left(1 + kl\dfrac{\exp(kl) + \exp(-kl)}{\exp(kl) - \exp(-kl)}\right)}\frac{\Delta H}{H}. \qquad (4)$$

Assuming continuity of H and Z, Young's modulus $Z(s)$ can be derived from Eq. 4 by calculating the increment ΔZ from the transfer impedance

$H(s)$ and using an approximation of k that is obtained by analyzing the data first in terms of a simple model consisting of damped and undamped elastic elements (Jung et al., 1988). Importantly, this model serves no other purpose than providing a starting point in the final calculation of the actual Young's modulus Z (De Winkel et al., 1993).

The time-resolved Young's modulus obtained from sinusoidal analysis

As set out above, the Laplace transform of the response to a single step yields the full frequency behavior of the mechanical transfer impedance and subsequently Young's modulus. Importantly, this method presupposes a linear relation between the tension and the applied length change and thus ignores any strain dependence of the Young's modulus. This problem is largely circumvented by a sinusoidal analysis. The development of the mechanical transfer impedance during a length change is obtained from the combination of the responses to a relative length change $\epsilon_{step}(t)$, $\epsilon_{step}(t) + \epsilon_{sin}(t)$, and $\epsilon_{step}(t) + \epsilon_{cos}(t)$, with $\epsilon_{step}(t)$ the length step and $\epsilon_{sin}(t)$ and $\epsilon_{cos}(t)$ the sinusoidal length modulations. The phase of the modulations is locked to the length step, with $\epsilon_{cos}(t)$ shifted about 90° relative to $\epsilon_{sin}(t)$. The measurement of two phase-shifted transients is necessary for determining the amplitude $|Z|$ of Young's modulus and its phase Φ_Z at each moment in time, independent of the modulation frequency. The net response to the modulation is now obtained from subtracting the response to the single step from that to the modulated signals. In this context it is important to note that the data are digitally sampled at a frequency of at least 20 times the modulation frequency. The distinct transients thus may be maximally shifted up to 0.1π radians relative to each other. Before the single step is subtracted from the modulated signal, the magnitude of the shift is determined and corrected for.

The net response to the modulation is now characterized by a time-dependent mechanical transfer impedance $H(s, t)$, reflecting the change in the Young's modulus of the fiber during the length step. In the analysis of $H(s, t)$ we consider linear and quadratic terms only. This is justified when the deviations from nonlinearity are small. Thus the mechanical transfer impedance as obtained from a sinusoidal modulation must be similar to that obtained from a small length step. Furthermore, the quadratic term in $H(s, t)$ should not be strain dependent, i.e., the change in Young's modulus should be proportional to the stretch. When these conditions are satisfied, the tension responses to the sinusoidal length changes $\epsilon_{sin}(t) = A_s \cos(\omega t + \vartheta)$ and $\epsilon_{cos}(t) = A_c \cos(\omega t + \varphi)$ are

$$\frac{T_s(t)}{A_s} = \frac{1}{2} H(s, t) e^{i(\omega t + \vartheta)} + \frac{1}{2} H^*(s, t) e^{-(\omega t + \vartheta)} \quad (5a)$$

$$\frac{T_c(t)}{A_c} = \frac{1}{2} H(s, t) e^{i(\omega t + \varphi)} + \frac{1}{2} H^*(s, t) e^{-i(\omega t + \varphi)}. \quad (5b)$$

This can also be expressed as

$$T_s(t)/A_s = \mathrm{Re}(H) \cos(\omega t + \vartheta) - \mathrm{Im}(H) \sin(\omega t + \vartheta) \quad (6a)$$

$$T_c(t)/A_c = \mathrm{Re}(H) \cos(\omega t + \varphi) - \mathrm{Im}(H) \sin(\omega t + \varphi). \quad (6b)$$

Now the norm $|H(s, t)|$ and the phase $\Phi_H(s, t)$ of the mechanical transfer impedance are found from Eq. 6:

$$|H(s, t)| = \sqrt{\frac{(T_c(t)/A_c)^2 + (T_s(t)/A_s)^2 - 2(T_s(t)T_c(t)/A_sA_c)\cos(\vartheta - \varphi)}{\sin^2(\vartheta - \varphi)}} \quad (7a)$$

and

$$\tan(\Phi_H(s, t)) = \frac{T_c(t)\cos(\omega t + \vartheta)/A_c - T_s(t)\cos(\omega t + \varphi)/A_s}{T_c(t)\sin(\omega t + \vartheta)/A_c - T_s(t)\sin(\omega t + \varphi)/A_s}. \quad (7b)$$

Equation 7 shows that $H(s, t)$ is defined at each time t when the tension transients T_c and T_s are known. Using only a single modulation, the amplitude and phase of H are extracted from the amplitude and phase shift over at least one modulation period. This consequently limits the time resolution at which H can be determined to the period of one oscillation. For a modulation frequency ω of 10 kHz this is 100 μs; however, for 250 Hz this increases to 4 ms. In contrast, the time resolution in both cases is limited by the sampling frequency when the two phase-shifted transients are used.

From $H(s, t)$ the time-resolved Young's modulus $Z(s, t)$ can be extracted, using Eq. 4, when the requirement of continuity of $H(s, t)$ and $Z(s, t)$ is satisfied. In this context it is important to realize that Young's modulus affects the resonance frequency of the fiber. This is reflected by the factor $(\exp(kl) - \exp(-kl))$ in Eqs. 1 and 2. Now a small change in Young's modulus Z results in a shift of this resonance frequency. Inspection of Eq. 4 reveals that in the range near the resonance frequency, the denominator becomes large. Consequently, a small change in Young's modulus Z results in a large change in H. This observation shows that in the vicinity of the resonance frequency the change in the elastic properties of the muscle fiber cannot simply be assessed by inspection of the ratio of the tension and length change (H). Rather, it underlines the necessity to extract Young's modulus from the experimental data.

RESULTS

The muscle fibers were stretched or released between 0.5 and 4 nm/half-sarcomere. Stretches up to 1.5 nm/half-sarcomere were carried out within 35 μs. However, the larger steps were slowed down to 200 μs to avoid damage to the muscle fibers. In addition, sinusoidal length modulations were applied with frequencies ranging from 250 Hz to 10 kHz, and an amplitude of 0.25 to 0.40 nm/half-sarcomere peak to peak. In Fig. 2 the length change of the sarcomeres is plotted as observed by HeNe diffraction. The plot shows that the length change, applied externally, is absorbed by the sarcomeres. The 10-kHz oscillation is clearly recognizable.

In Fig. 3 *a*, a stretch of 1 nm/half-sarcomere applied to the muscle fiber is shown with and without a modulation of 10 kHz. The difference between these two curves yields the net oscillation with only a minimal distortion at the moment of the length step (Fig. 3 *c*). This indicates that the loudspeaker motor and position detection behave linearly. In contrast, the net tension response of a rigor muscle fiber (Fig. 3 *d*) shows a clear increase in amplitude after the stretch. Fig. 3, *b* and *d*, also shows that the tension oscillates nonsymmetrically around the tension response to a single step. This must be attributed to drift of the force transducer on a minute time scale as the transients with and without the modulation are taken separately.

From the tension transients in Fig. 3 the mechanical transfer impedance $H(s, t)$ and subsequently the time-resolved Young's modulus $Z(s, t)$ were extracted (Fig. 4). To check if the sinusoidal modulation changes the elastic properties of the rigor fiber, we compared Young's modulus

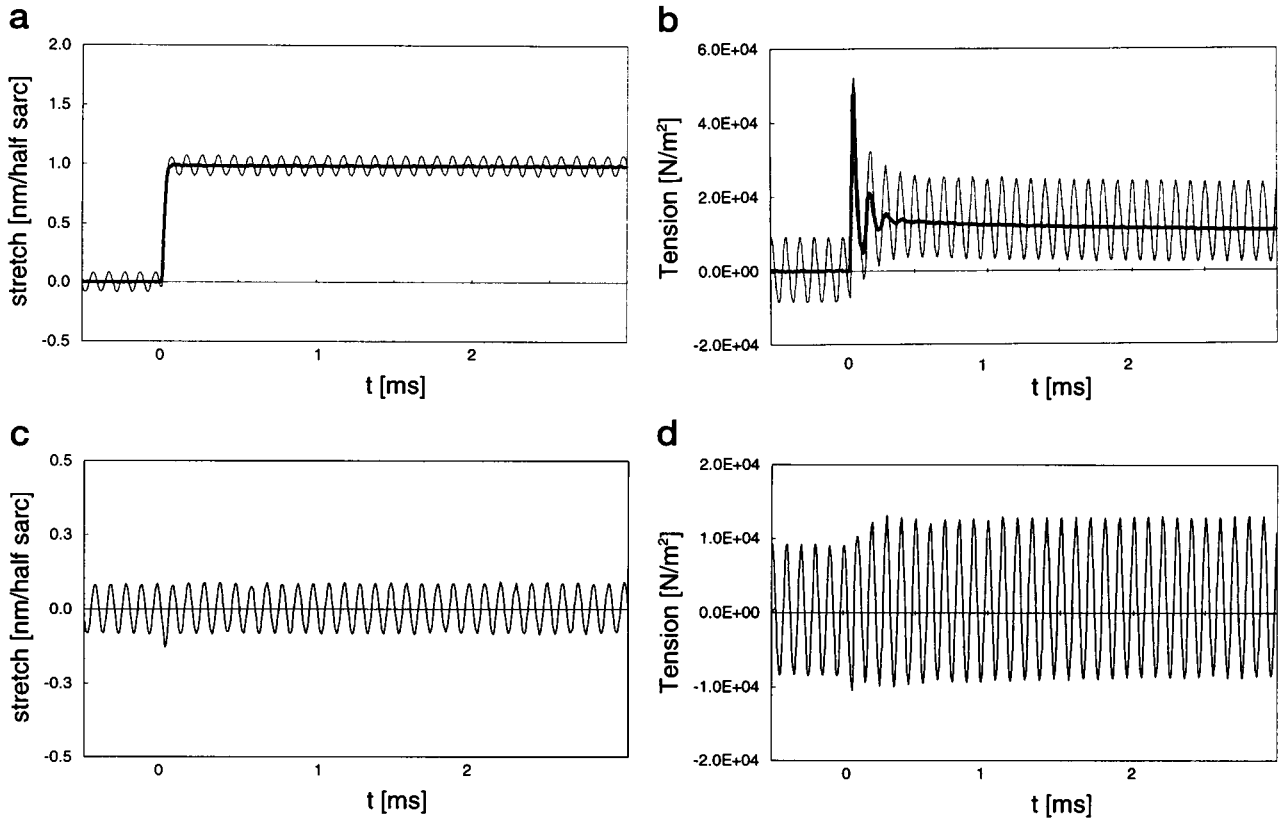

FIGURE 3 (*a*) The length step of 1 nm/half-sarcomere (thick) and of the same length step plus a 10-kHz modulation of 0.25 nm/half-sarcomere top/top (thin). (*b*) The tension responses corresponding to the length steps of *a*. (*c*) The net length modulation. (*d*) The net tension response.

before the length step with the value obtained from small length steps using the method described previously (De Winkel et al., 1993). The result from the sinusoidal modulation was found to lie consistently between the values obtained from a stretch and a release. This indicates that the modulation of 0.25 nm/half-sarcomere peak to peak does not significantly distort the properties of the fiber.

The time-resolved Young's modulus $Z(s, t)$ increases within 0.5 ms upon a stretch of the muscle fiber over the frequency range from 250 to 10,000 Hz. Similarly, it decreases upon a release. No appreciable relaxation to the original value was found in the 500 ms after the length change. The length steps larger than 2 nm/half-sarcomere yielded a similar response, although the fast components in the tension transient were suppressed because the steps were carried out much more slowly. The relative change in Young's modulus was found to depend linearly on the relative length change (Fig. 5). This indicates that it is justified to consider only the linear and quadratic terms in the data analysis. At frequencies below 1000 Hz the relative change in Young's modulus is about 20% per nm/half-sarcomere length change. Interestingly, the size of the effect decreases to about 10% per nm/half-sarcomere length change at 10 kHz (Fig. 6). The decrease is quite clear when comparing the results at the full frequency range of each fiber. However, because of variations between the preparations, the error margins in Fig. 6 are relatively large. Nevertheless, the difference between the strain dependence at 1 and 10 kHz was found to be significant (more than 99% confidence level, paired Student's *t*-test). Results at higher frequencies could not be obtained because the loudspeaker motor does not permit a continuous oscillation at frequencies higher than 10 kHz.

The change in Young's modulus was also studied in rigor buffer with 20 mM BDM (2,3-butadione 2-monoxime). BDM maintains sarcomere uniformity (Higuchi et al., 1995). We found no difference between Young's modulus in rigor with and without BDM. Furthermore, BDM did not influence the change in the Young's modulus upon a stretch or a release (Fig. 5). This confirms that inhomogeneities in the preparation can be excluded as an explanation for the observed effects. The above leads us to the conclusion that the strain dependence of the Young's modulus must be considered an intrinsic property of the skeletal muscle fiber in rigor.

DISCUSSION

Stretch and release experiments carried out on skinned single fibers of frog skeletal muscle under rigor conditions indicate that the elastic properties of the fiber depend on

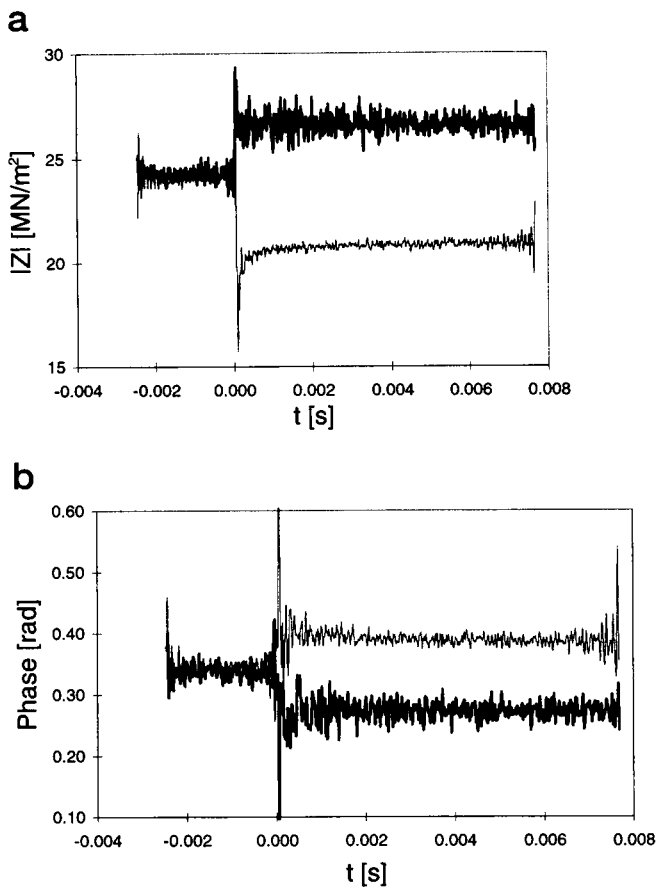

FIGURE 4 The time-resolved Young's modulus at 10 kHz as obtained from a stretch of 1.0 nm/half-sarcomere (thick) and from a release of 1.0 nm/half-sarcomere (thin). (*a*) The norm |*Z*|. (*b*) The phase Φ_Z.

strain. For modulation frequencies below 1000 Hz, the results show an increase in Young's modulus of 20% upon a stretch of 1 nm/half-sarcomere, consistent with various other studies (Kawai and Brandt, 1976, 1980; Higuchi et al., 1995). Importantly, the strain dependence of Young's modulus decreases at higher frequencies to about 10% upon a 1

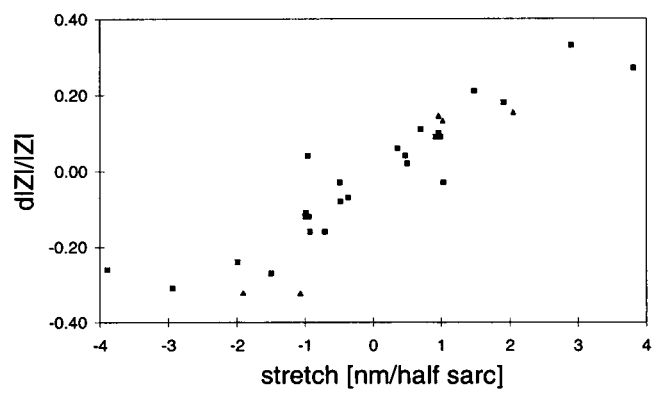

FIGURE 5 The relative change of the 10-kHz Young's modulus as a function of the relative length change. ■, Standard rigor. ▲, Rigor + 20 mM BDM.

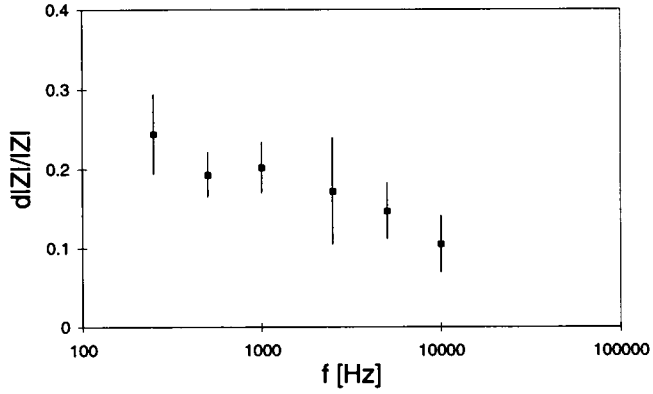

FIGURE 6 The relative change of the Young's modulus per nm/half-sarcomere stretch as a function of the modulation frequency.

nm/half-sarcomere stretch at a modulation frequency of 10 kHz.

As argued above, we believe that the strain dependence of Young's modulus and its frequency characteristic must be considered an intrinsic property of the muscle fiber in rigor. The Young's modulus of the fiber reflects the number of cross-bridges attached to the actin filaments, their elastic properties, and the elasticity of the actin filaments and other passive components in the muscle fiber. Consequently, for the molecular mechanism underlying the strain dependence, several possibilities must be considered.

Slackening of compliant in-series elements, such as the filaments, results in a loss of stiffness upon shortening. Indeed, slackening is observed in elastic materials such as rubber. However, when a certain fraction of the elastic elements slackens, this should result in a proportional decrease in stiffness over the full frequency range. This is at odds with our observations. To account for the decline of the effect at higher frequencies, the structures should slacken at a range of time scales. Thus some elements should only lose their stiffness during a slow release and maintain it during an equally large release carried out at a larger speed. This requirement is difficult to reconcile with the conventional idea of slackening.

Recently the increase in compliance of rigor fibers at low tension was attributed to actin filament compliance (Higuchi et al., 1995). In a separate study we discussed the effect of filament compliance on the interpretation of the elastic properties of skeletal muscle fibers (Blangé et al., 1996). This study showed that the elastic properties of partially activated fibers were described consistently in terms of a constant elasticity of the actin filaments E_f = 44 MN/m^2 and a cross-bridge stiffness increasing linearly with tension. Assuming that the elastic properties of the actin filament are not influenced by ATP or calcium, the same constant value holds under rigor conditions. More importantly, we believe that a strain-dependent actin filament compliance cannot account for the frequency behavior of the strain dependence of the rigor Young's modulus: at frequencies above 40 kHz, the Young's modulus is larger than 40 MN/m^2, reflecting a

small total compliance. Consequently, the actin filaments make up a large fraction of the total compliance of the half-sarcomere. However, at lower frequencies the Young's modulus of the fiber is substantially smaller, reflecting a much larger compliance. The share of the actin filament compliance in the total therefore decreases strongly (Blangé et al., 1996). If the elastic properties of the actin filament were strain dependent, this should be revealed most clearly under those circumstances in which the actin filament compliance makes up a large fraction of the total compliance, thus at high frequencies. Interestingly, we find precisely the opposite: the strain dependence is exhibited most clearly at the frequencies below 1000 Hz, where the contribution of the actin filament compliance is relatively small. Therefore, we believe that a strain-dependent actin filament elasticity cannot account for the frequency characteristic of the strain dependence of Young's modulus in rigor skeletal muscle.

The strain dependence of Young's modulus cannot be explained in terms of the number of strongly bound cross-bridges. Following this reasoning, a stretch of up to 4 nm/half-sarcomere would result in an increase of up to 40% in the number of strongly bound cross-bridges (Fig. 5). This explanation is not realistic. Furthermore, it is again difficult to understand why Young's modulus would exhibit a smaller change at high frequencies than at low frequencies.

The above brings us to the conclusion that passive structures outside the cross-bridges are not the likely cause of the observed effect. A more conceivable explanation can be found in the properties of the cross-bridges themselves. Several studies suggest that negatively strained cross-bridges are less stiff than positively strained ones (Kawai and Brandt, 1976; Higuchi et al., 1995). Our results imply that the difference in stiffness becomes smaller toward higher frequencies. Several hypotheses can explain these observations. They all have in common that transitions must occur between distinct rigor states, but various possibilities must be considered regarding their nature.

One explanation is that the cross-bridge "buckles" upon shortening and acquires a smaller low-frequency Young's modulus, whereas it is retained at high frequencies. This is consistent with the view presented in other studies (Kawai and Brandt, 1976; Higuchi et al., 1995), although the transition is no simple slackening, but rather a complicated change in the viscoelastic properties of the globular protein. The frequency dependence of the Young's modulus in both the "buckled" and the normal state can be attributed to the viscoelastic properties of the protein and can be considered an effect similar to "stress relaxation."

A second explanation is that there are two distinct rigor states with different equilibrium strains but essentially the same viscoelastic properties. This may be similar to the model proposed by Huxley and Simmons (1971). However, one can also think of a dissociation/reassociation of rigor cross-bridges with restrictions to double head attachment (Tözeren and Schoenberg, 1986; Tözeren, 1987). Upon a length change a redistribution of the cross-bridges takes place toward the state with the most favorable equilibrium strain. When such transitions can occur during length changes, the effective stiffness is smaller than when the transitions do not occur, because the transition allows an extra pathway for the cross-bridge to comply with the length change. As such a transition will take some time, they can only occur during frequency modulations at a sufficiently low frequency. During high-frequency modulations the cross-bridges cannot accomplish such a transition, so they will experience the stiffness corresponding to a unique strongly bound state. This explains the frequency characteristic of Young's modulus. A strain dependence of the low-frequency Young's modulus can occur when such transitions are less likely for positively strained cross-bridges than for negatively strained ones.

The authors wish to acknowledge Prof. M. Kawai (University of Iowa) for many helpful discussions and Dr. F. A. M. van Kaam (University of Amsterdam) for improving the low-frequency performance of the motor.

This study was supported by the Dutch Foundation for Life Sciences (NWO).

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